Localization of chitin synthetase in cell-free homogenates of Saccharomyces cerevisiae: Chitosomes and plasma membrane

(Specific density/1,3-β-glucan synthetase/microvesicles/vesicles/zymogen)

Carlos A. Leal-Morales†‡, Charles E. Bracker‡, and Salomon Bartnicki-Garcia*

*Department of Plant Pathology, University of California, Riverside, CA 92521; and ‡Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907

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ABSTRACT We describe an improved method for fractionating cell-free extracts of Saccharomyces cerevisiae to separate its membranous components by a combination of isopycnic and velocity sedimentations. These procedures were used to examine the subcellular distribution of chitin synthetase (chitin-UDP acetylglucosaminyltransferase; EC 2.4.1.16) in homogenates from exponentially growing walled cells of a wild-type strain of yeast. Chitin synthetase (Chs1) activity was mainly found in two distinct vesicle populations of nearly equal abundance but with markedly different buoyant densities and particle diameters. One population contained 45–65% of the total chitin synthetase and was identified as chitosomes because of the characteristic low buoyant density (1.15 g/ml); it also lacked 1,3-β-glucan synthetase activity. The second population (35–55%) was identified as plasma membrane because of its high buoyant density (1.22 g/ml), large vesicle size (median diameter = 252 nm), and presence of vanadate-sensitive ATPase. This fraction cosedimented with the main peak of 1,3-β-glucan synthetase. A third, minor population of chitin synthetase particles was also detected. Essentially all of the chitin synthetase in the two vesicle populations was zymogenic; therefore, we regard these vesicles as precursors of the final active form of chitin synthetase whose location in the cell has yet to be unequivocally determined.

Because of the importance of chitin synthetase (chitin-UDP acetylglucosaminyltransferase; EC 2.4.1.16) in cell wall formation and morphogenesis in fungi, considerable attention has been devoted to the characterization of this enzyme and the cell biological mechanisms by which it functions. Conflicting claims have been made about the cellular localization of chitin synthetase: some studies emphasize the localization in chitosomes (1–4); others maintain it is mainly, if not exclusively, a plasma membrane-bound enzyme (5–7). Saccharomyces cerevisiae (2, 5) has been a salient subject in this controversy.

Our recent experience with improved methods for separating membranous organelles by density gradient sedimentation (8) was applied to examine the intracellular distribution of chitin synthetase in cell-free homogenates of S. cerevisiae. We used exponentially growing, walled cells rather than protoplasts (4, 5) since the former represent a normal morphogenetic state of this organism and thus offer a greater potential for revealing the in vivo distribution of chitin synthetase.

METHODS

Cultivation and Cell Disruption. S. cerevisiae (ATCC 26109) was grown in 1200 ml of filter-sterilized medium (0.7% Difco yeast nitrogen base; 2% glucose) (9) in a shaker bath at 30°C for 12 hr. Cells were harvested in logarithmic phase (35–40 × 10⁶ cells per ml) by centrifugation at 1500 × g for 5 min, washed with 1 mM sodium EDTA (pH 4.2), resuspended in 20 ml of 1 mM EDTA, and filtered. Cells (3.9 g of wet weight) were mixed with 8 ml of 0.5 to 0.45-mm glass beads and 4 ml of 1 mM EDTA in 17% sucrose and disrupted in a Braun MSK homogenizer for 12 sec. The homogenate was diluted 1:1 with 1 mM EDTA and centrifuged at 1500 × g for 10 min. The supernatant, which contained about 90% of the total chitin synthetase activity, was the crude homogenate used below.

Density Gradient Centrifugation. For the first isopycnic sedimentation, 6.6 ml of crude homogenate was layered on top of a 32-ml linear sucrose gradient (10–65% [wt/vol]) in 30 mM Tris-HCl buffer (pH 7.5) and centrifuged at 265,000 × g (Rₑᵥ) for 2 hr in a Beckman 70 Ti rotor. The gradient was fractionated (1 ml) from the top with an ISCO fractionator. Peak samples were pooled, diluted 1:1 with Tris buffer, layered onto 10-ml linear sucrose gradients (10–65% [wt/vol]) in Tris buffer, and recentrifuged under two different conditions: (i) in a Beckman 70.1 Ti rotor for 3 hr at 247,000 × g (Rₑᵥ) for the second isopycnic sedimentation and (ii) in 300,000 × g (Rₑᵥ) for isolation of the plasma membrane.

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a Beckman SW-41 Ti rotor for 3 hr at 81,000 × g (R$_{av}$) for velocity sedimentation.

**Enzyme Assays.** Chitin synthetase activity was measured as described (10) except that 10% trichloroacetic acid was added to the assay mixtures before filtration (9). The assay mixture (125 µl) contained 80 µg (736 N$^\circ$-benzoyl-L-arginine ethyl ester units) of trypsin per ml but no phosphatidylserine. Activity is expressed in units (nmol of GlcNAc per min). β-Glucan synthetase activity was measured as described (11) except that 5 mM ATP was present in the incubation mixture. Activity is expressed in units (nmol of glucose per min). ATPase was measured in a 300-µl reaction mixture containing 15 mM MgCl$_2$ and 15 mM ATP in 70 mM Mes/Tris buffer, pH 6.7. Where indicated, 5 mM sodium azide or 1.6 mM sodium vanadate was included. After 1 hr of incubation, ADP was determined by HPLC on an AX-10 Varian column eluted at 2 ml/min with 0.3 M KH$_2$PO$_4$/KOH, pH 6.5.

**Electron Microscopy.** Samples for electron microscopy were prepared by negative staining as detailed elsewhere (1) except that fixation with glutaraldehyde was omitted.

**RESULTS**

**First Isopycnic Sedimentation.** During a single isopycnic centrifugation on a 10–65% sucrose gradient, the chitin synthetase in the crude homogenate separated into two distinct peaks (Fig. 1). Peak A with low buoyant density (1.150 g/ml) was the presumptive chitosomal fraction; peak B with a higher density (1.219 g/ml) was the presumptive plasma membrane fraction.

Overall recovery of chitin synthetase activity in the gradients was good (87% of the activity applied). In three separate experiments, the proportion of chitin synthetase detected in the two peaks varied from 45% to 65% in peak A and from 35% to 55% in peak B. In both peaks, the vast majority of the enzyme was zymogenic and required limited proteolysis for activation. There was only a small amount of active chitin synthetase in these fractions (activity detected without trypsin treatment): 1.4% of the total activity in peak A and 6.8% in peak B. The chitin synthetase in both peaks was strongly inhibited by 3 mM Co$_2^{2+}$; the activity of peak A decreased 95%; that of peak B decreased 83%.

The 1,3-β-glucan synthetase activity of the cell homogenate also separated into two distinct peaks; a minor one with a density of 1.175 g/ml (Fig. 1, peak C), which coincided with a shoulder of chitin synthetase peak A, and a major one (peak D) at 1.219 g/ml, which coincided with peak B of chitin synthetase.

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**Fig. 2.** Electron micrographs of peak fractions after one (Fig. 1) or two (Fig. 4) isopycnic centrifugations. (A) Sample from the chitosomal peak (Fig. 1, peak A) after a single isopycnic centrifugation. It consists of some microvesicles (C), a few larger vesicles (V), and a lot of small background debris (D). (B) Sample from the plasma membrane peak (Fig. 1, peak B/D) after a single isopycnic centrifugation. Large membrane vesicles (V) on a background of small debris. (C) Sample from the chitosomal peak after a second isopycnic centrifugation (Fig. 4, peak A). It contains both vesicles and microvesicles. (D) Sample from the plasma membrane peak after a second isopycnic centrifugation (Fig. 4, peak B). (Bars = 200 nm.)
The UV-absorbance profile (Fig. 1) shows three broad peaks, two of which closely parallel the peaks of chitin and 1,3-β-glucan synthetase. However, most of this absorbance probably corresponds to the contaminating particles shown in Fig. 2 A and B.

ATPase activity cosedimented with peaks A and B in Fig. 1. The activity of peak A was substantially inhibited by sodium azide (43%) but only weakly inhibited by sodium vanadate (16%). Conversely, the ATPase of peak B was 48% inhibited by vanadate but not (2%) by azide.

Electron microscopic examination of the peak fractions of chitin synthetase showed vesicles among a background of unrecognizable fine particulate matter. Peak A contained mainly microvesicles (70% were 40–70 nm in diameter) (chitosomes) and some larger membrane vesicles (16% were >100 nm in diameter) (Fig. 2A). Conversely, peak B contained mainly large vesicles with a smooth profile (71% were 100–325 nm in diameter) and a smaller proportion of microvesicles (27% were <100 nm) (Fig. 2B).

Second Isopycnic Sedimentation. The two fractions with highest activity from each of the two peaks of chitin synthetase (Fig. 1, peaks A and B) were pooled, diluted 1:1 with Tris buffer, and recentrifuged for 3 hr at 247,000 × g (R$_{av}$) in an attempt to further separate and identify structures not resolved in the first isopycnic centrifugation. This second, shorter isopycnic centrifugation yielded single sharp peaks of chitin synthetase and 1,3-β-glucan synthetase at the expected buoyant densities: 1.143 and 1.196 g/ml for chitin synthetase (Fig. 4) and 1.168 and 1.208 g/ml for 1,3-β-glucan synthetase (not shown). With this shorter centrifugation, contaminating fine particles did not reach isopycnic equilibrium (see UV-absorbance profiles in Fig. 4) and were thus effectively separated from vesicles that did. The resulting peak fractions of chitin synthetase consisted almost entirely of vesicles. The presumptive chitosomal peak contained a mixture of small (53% were <100 nm) and large vesicles (47% were 100–200 nm) (Fig. 2C). The presumptive plasma membrane fraction contained mainly large vesicles (68% measured 100–325 nm) (Fig. 2D).

Velocity Sedimentation. The presence of a mixed vesicle population in the presumptive chitosome peak (Fig. 1, peak A; Fig. 2A) even after the second isopycnic sedimentation (Fig. 2C) prompted us to test if a different sedimentation based on velocity would separate vesicles according to size. This was the case. When a pool of peak A fractions was centrifuged on a linear sucrose gradient in a swinging bucket rotor (Beckman SW-41 Ti) for 3 hr at 81,000 × g (R$_{av}$), chitin synthetase activity split into two peaks, a major slow-moving
DISCUSSION

Basis for Identifying Two Chitin Synthetase Populations as Chitosomes and Plasma Membrane. We have shown that the chitin synthetase activity in a cell-free homogenate of *S. cerevisiae* resides mainly in two populations of vesicles that differ markedly in buoyant density and size. One population was identified as chitosomes because of microvesicular size and characteristic low buoyant density. It also lacked 1,3-beta-glucan synthetase activity. The second one had the high buoyant density typical of plasma membrane of *S. cerevisiae* (12, 13), contained vanadate-sensitive ATPase, and cosedimented with the main fraction of 1,3-beta-glucan synthetase, an enzyme believed to be associated with the plasma membrane (14, 15). Because of their characteristic and drastically different buoyant density and the absence of 1,3-beta-glucan synthetase, it is unlikely that the microvesicles are fragments of the larger vesicles.

Although our present knowledge prompts us to identify the microvesicles as chitosomes and the larger vesicles as plasma membrane, we should bear in mind that these were structures isolated from a cell homogenate. Until high-resolution immunocytochemistry or equivalent evidence on chitin synthetase is available, the true significance—*in vivo*—of the chitin synthetase structures found *in vitro* cannot be decided.

Basis for Separating the Vesicle Populations. By selecting appropriate centrifugation conditions, we were able to separate two major and one minor population of vesicles containing chitin synthetase from cell homogenates of *S. cerevisiae*. It was important to combine centrifugations that separated particles according to different criteria. The first isopycnic centrifugation yielded discrete but heterogeneous populations of vesicles of similar buoyant density but widely different size plus other smaller UV-absorbing particles. Subsequent recentrifugations were designed to separate particles mainly by their differences in sedimentation velocity.

Proper sample handling between centrifugations was also critical to preserve enzyme activity. Thus, as we transferred samples from one centrifugation step to the next, there was a need to reduce the initial sucrose concentration, but dialysis and ultrafiltration both caused severe loss of activity. A limited dilution was the only effective way to lower sucrose concentration and retain enzyme activity.

Because of discrepant buoyant density, the separation of membranous organelles poses a unique case for each organism. Thus, the conditions we selected for successful separation of vesicle types in *S. cerevisiae* need not apply to other fungi. For instance, in *Mucor rouxii*, a single isopycnic centrifugation of a crude cell-free supernatant was sufficient to separate chitosomes from most larger vesicles (16).

Reconciling Divergent Views on Subcellular Distribution of Chitin Synthetase. Although evidence for a low-buoyant-
density, or slowly sedimenting form of chitin synthetase (i.e., chitosomes) appears in most studies on the localization of this enzyme in *S. cerevisiae* (2, 5, 9, 17), its significance is sometimes overlooked and emphasis is placed on the heavy form (plasma membrane). Yet, the relative abundance of plasma membrane-bound chitin synthetase has been controversial. Whereas Cabib and colleagues (5, 9) reported that most activity was in the plasma membrane of *S. cerevisiae* protoplasts, Hanson and Marple (22) found only 5–10% was associated with plasma membrane, and, under conditions that prevented vacuole lysis during protoplast rupture, Schwencke et al. (4) found that the main portion of chitin synthetase was in chitosomes. They also recognized that the relative proportion of chitin synthetase in plasma membrane and chitosomal membranes may be affected by differential losses during purification. We believe our procedure gives a more reliable picture of the subcellular distribution of chitin synthetase in yeast. By using growing, walled cells rather than protoplasts and by separating organelles in two centrifugation steps with minimum handling, we reduced the possibility of structural artifacts, recovered most of the initial enzyme activity, and obtained fairly homogenous subcellular fractions. Accordingly, we conclude that chitin synthetase in cell-free extracts of wild-type yeast exists in two different vesicle populations of nearly equal abundance: chitosomes and plasma membrane.

**Significance of Two Vesicle Populations.** Although the existence of two separate major populations of chitin synthetase-containing vesicles seems well proven, their cytological relationship remains a point of speculation. As suggested earlier (18), chitosomes could be the delivery vehicle of chitin synthetase to its ultimate destination at the cell surface (plasma membrane). But inasmuch as the two particle populations carryzymogenic chitin synthetase, both are potential precursors to a final active form. The location of the final active form could not be established here because active enzyme was virtually absent in the homogenate (90–95% of the enzyme detected was zymogenic).

Recently, two different genetic loci were found associated with chitin synthetase in yeast (19, 20). By the criteria of zymogenicity and sensitivity to cobalt (20, 21), the chitin synthetase in the two vesicle populations we separated—chitosomes and plasma membrane—appears to be of the Chs1 type. On the basis of evidence and genetically altered strains, the essentiality of Chs1 for chitin synthesis *in vivo* has been questioned (19–21). However, we think it is premature to discount the role of this chitin synthetase in the physiology of *S. cerevisiae*, particularly since it comprises by far most of the chitin synthetase activity recovered from exponentially growing cells. The multiplicity of genetic loci for chitin synthetase poses the immediate question of whether chitin synthetases encoded by different genes have the same subcellular distribution. Our preliminary experiments on Chs2, done on a yeast strain (D1B; see ref. 20) with a disrupted Chs1 gene (*his chs1::URA3*), indicated that this second kind of chitin synthetase is also distributed in two major subcellular fractions with different buoyant densities and sedimentation velocities.6

6Due to lower amounts and greater instability of the chitin synthetase present in this mutant, we have not yet been able to confirm whether the two vesicular fractions with Chs2 activity are identical to those described here for Chs1.

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